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REGULAR ARTICLE

Roles of activated astrocyte in neural stem cell proliferation and differentiation

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Abstract Recent studies demonstrated that the molecules secreted from astrocytes play important roles in the cell fate determination of neural stem cells (NSCs). However, the exact molecules involved and its possible mechanisms in the process remain largely unknown. In this study, astrocyte-conditioned medium (ACM) obtained from astrocytes unstimulated or stimulated by lipopolysaccharide was prepared to treat NSCs. The results showed that both the proliferation and differentiation of NSCs treated with stimulated ACMs were significantly increased compared with those treated with unstimulated ACM. Interleukin-6 (IL-6) antibody neutralization of the ACMs decreased NSC proliferation and astrogliogenesis, while NSC neurogenesis was increased. In contrast, recombinant IL-6 cytokine increased NSC proliferation and astrogliogenesis, but decreased neurogenesis. Furthermore, the expression of phosphorylated signal transducer and activator of transcription 3 (p-stat3) protein as well as serial of basic helix–loop–helix transcription factors (bHLH) mRNA in NSCs exposed to stimulated ACMs significantly increased, respectively. The expression levels of p-stat3 protein and bHLH mRNA of NSCs were significantly altered after adding anti-IL-6

Abbreviations: ACM, astrocyte-conditioned medium; BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; bHLH, basic helix–loop–helix transcription factors; BMP, bone morphogenetic protein; CNS, central nervous system; CNTF, ciliary neurotrophic factor; DAPI,4, 6-diamino-2-phenylindole; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; IL-1β, interleukin-1 beta; IL-6, Interleukin-6; JAK/STAT, Janus kinase/signal transducer and activation of transcription; LPS, lipopolysaccharide; MAP2, microtubule-associated protein 2; MTT, (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; NSCs, neural stem cells; N-ACM, normal ACM; p-stat3, phosphorylated stat3; TNF-α, tumor necrosis factor-alpha.

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antibody or recombinant IL-6, respectively. The data suggest that IL-6 secreted from activated astrocytes participates in ACM-induced proliferation and differentiation of NSCs via the phosphorylation of stat3 signals and the expression of bHLH transcription factors.

Introduction

Because of the potential of self-renewal and multilineage differentiation of neural stem cells (NSCs), transplantation of NSCs has been considered to be a promising therapy for neural disorders (Blurton-Jones et al., 2009; Wu et al., 2010). However, some studies have shown that proliferation of the transplanted NSCs is limited with most of them differentiating into astrocytes in damaged central nervous system (CNS). Maintaining the self-renewal capacity of NSCs and controlling their directed differentiation into specific neural cell types are therefore major challenges in using NSCs for neural treatment. Less clear are the molecular mechanisms that regulate the proliferation and differentiation of NSCs and how these might affect the neurogenesis of NSCs under pathologinal conditions. Recent studies demonstrated that in addition to the intrinsic properties of stem cells, local microenvironment or "niche", such as growth factors, cytokines and cell–cell contact, plays key roles in the cell fate determination of stem cells (Ma et al., 2005; Jiao and Chen, 2008; Moyse et al., 2008).

It is well documented that astrocytes constitute a major component of neural microenvironment in the CNS (Ling and Leblond, 1973). In addition to providing nutrition, support and protection to neurons under physiological conditions, astrocytes play crucial roles in the CNS pathologies. There is growing evidence that injuries or diseases in the CNS would not only elicit a characteristic inflammatory reaction but also enhance proliferation and differentiation of NSCs (Wang and Shuaib, 2002; Schwab and McGeer, 2008). Astrocytes, as one of the key players mediating inflammatory response, were markedly activated in various CNS diseases and produced pro- and anti-inflammatory cytokines, trophic factors and chemokines (Ridet et al., 1997; Lau and Yu, 2001), which participated in neuronal survival, maturation and neurogenesis (Song et al., 2002; Emsley et al., 2004). This indicates that astrocytes may affect proliferation and differentiation of NSCs in the injured CNS.

Previous studies have demonstrated that the effects of stem cell transplantation in animal experiments vary with different times of cell grafting (Chen et al., 2001; Hofstetter et al., 2002). One possible explanation for this may be that the tissue microenvironment at the site of stem cell transplantation changes along with time after injury and this would cause different behaviors of transplanted cells. Therefore, unraveling the molecular signals underlying NSCs and niche interactions would provide valuable insight into potential usage of NSCs for neural treatment. Considering the pivotal roles of astrocytes in neurogenesis (Jiao and Chen, 2008; Song et al., 2002) and our previous report that molecules secreted from activated astrocytes during different stages of inflammation affected bone marrow-derived mesenchymal stem cells in different manners (Wang et al., 2009), it remains to be elucidated if molecules derived from astrocytes with distinct inflammatory states would likewise regulate the cell fate determination of NSCs.

It is well documented that activation of astrocytes and its related functions *in vivo* is a complex process which may involve different stimulating factors. In the present study, astrocytes were stimulated by lipopolysaccharide (LPS), a potent inflammatory activator of astrocytes, to mimic partially the astrocytic reactions following an inflammatory injury *in vivo*. NSCs were then incubated with conditioned medium from LPS-stimulated astrocytes to determine the effects of inflammation-activated astrocytes on the proliferation and differentiation of NSCs and possible molecular signals involved in the process *in vitro*. The findings would help explain on how the functional changes of astrocytes in different phases of CNS diseases or injuries would affect the stem cells. The study would also provide a cellular and molecular basis for designing and selecting a proper time window for an effective NSC transplantation in clinic.

Results

Effects of different astrocyte-conditioned mediums (ACMs) on the proliferation of NSCs

To test the effects of ACMs derived from LPS-activated astrocytes on the proliferation of NSCs, MTT assay and BrdU incorporation analysis were performed. The results showed that both the cell viability and BrdU incorporation of NSCs were significantly increased in ACM-treated groups compared with the DMEM/F12-treated group (Fig. 1). Furthermore, the cell viability of NSCs was increased in 36 h ACM (0.3318 ± 0.03) and 72 h ACM (0.3103 ± 0.03) groups in comparison with that of N-ACM (0.2827 ± 0.02) and the difference between N-ACM and 36 h ACM was statistically significant ($p < 0.05$). Similarly, the BrdU incorporation of NSCs (Fig. 1B,C) was also increased in 36 h ACM (24.17 ± 2.7) and 72 h ACM (21.39 ± 2.9) groups compared with that of N-ACM (18.26 ± 2.6) and there was statistically significant difference between N-ACM and 36 h ACM ($p < 0.05$). Both the results of MTT and BrdU incorporation assays indicated some mitogenic factors released from LPS-activated astrocytes could promote NSCs proliferation. Additionally, the effects of 36 h ACM ($p < 0.01$) and 72 h ACM ($p < 0.05$) on both NSC viability and BrdU incorporation were significantly greater than that of 12 h ACM, respectively, suggesting that the molecules produced by astrocytes varied at different activated states causing distinct effects on NSCs proliferation. Because ACM contained LPS, we tested if residual LPS in ACM would be responsible for the proliferation and differentiation of NSCs. In this connection, NSCs were cultured with the same concentration of LPS as ACM. There was no significant difference between LPS- and DMEM/F12-treated NSCs (0.2497 ± 0.03 vs 0.2518 ± 0.02) as shown by cell viability analysis. Similarly, there was also no significant difference between LPS- and DMEM/F12-treated NSCs in the BrdU incorporation results (13.74 ± 2.8 vs 13.08 ± 3.1) (Fig. 1), suggesting that residual LPS does not directly stimulate NSC's

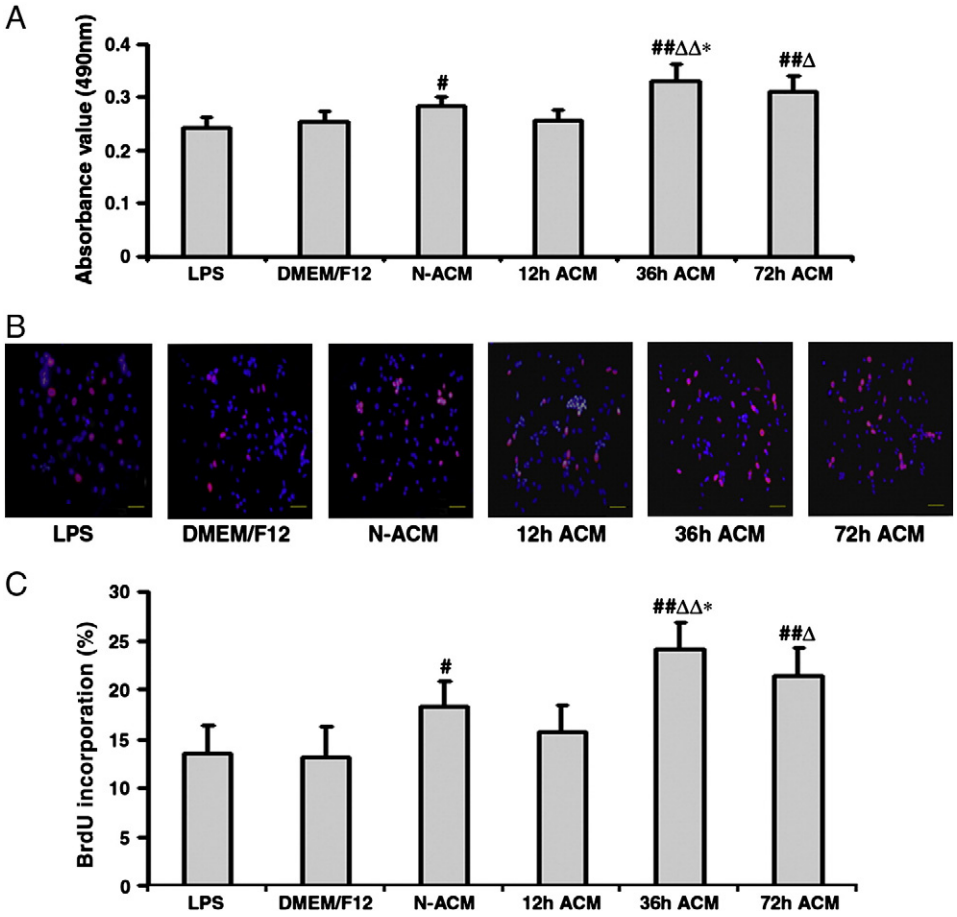

Fig. 1 ACM promotes NSC proliferation. NSCs were cultured in LPS-containing DMEM/F12 (LPS), DMEM/F12 alone, N-ACM, 12 h, 36 h, and 72 h ACM (without dilution), respectively, with 5 ng/ml bFGF for 2 days and then detected for cell proliferation. A) MTT assay shows the cell viability. B) Showing the representative BrdU immunofluorescence images in LPS, DMEM/F12, N-ACM, 12 h, 36 h, and 72 h ACM group, respectively. The cell nuclei were counterstained with DAPI. BrdU (red), DAPI (blue). C) Quantification of BrdU-positive NSCs over the total DAPI-positive cells. Each bar represents mean ± SD ($n=4$). Scale bar: 50 μm. #$P<0.05$, ##$P<0.01$ vs DMEM/F12; *$P<0.05$ vs N-ACM; Δ$P<0.05$,Δ Δ $p<0.01$ vs 12 h ACM.

proliferation, which is consistent with previous studies(Cacci et al., 2008; Monje et al., 2003).

Effects of different ACMs on the neuronal differentiation of NSCs

Previous studies have reported that astrocytes play important functions in the differentiation of NSCs (Song et al., 2002; Emsley et al., 2004; Faijerson et al., 2006). To investigate whether the activated astrocytes at different inflammatory states have distinct roles in neurogenesis, NSCs were exposed to different ACMs for 7 days and then harvested. Immunostaining and Western blot were conducted to examine the expression of MAP2, a specific neuronal marker. Firstly, the results (Fig. 2) showed that ACM significantly increased the expression of MAP2 in induced NSCs compared with DMEM/F12 alone. Secondly, as shown in Fig. 2A,B, the percentage of MAP2-positive cells was significantly increased in 36 h (31.09 ± 3.1, $p<0.01$) and 72 h ACM (27.57 ± 2.9, $p<0.05$) groups compared with that in N-ACM group (22.64 ± 2.6). The proportion of MAP2-positive NSCs exposed to 36 h ACM and 72 h ACM was significantly higher than that of 12 h ACM (19.35 ± 2.8, $p<0.01$). This was corroborated by Western blot results (Fig. 2D) which showed that MAP2 protein expression in both 36 h and 72 h ACM groups was more pronounced than N-ACM. MAP2 expression in 12 h ACM-treated NSCs was the weakest compared with other ACM groups. In addition, the results showed that compared with DMEM/F12, the LPS alone did not directly affect NSC's neuronal differentiation (16.44 ± 2.8 vs 17.18 ± 2.7, $p>0.05$).

Effects of different ACMs on the astrocytic differentiation of NSCs

To further explore whether activated astrocytes at different inflammatory stages regulate the astrogliogenesis differently, we investigated the expression of GFAP, a specific astrocytic marker. The results (Fig. 2) also showed that ACMs increased the expression of GFAP compared with DMEM/F12 alone. Moreover, as shown in Fig. 2A,C, the percentage of GFAP-positive cells was increased in NSCs exposed to inflammatory ACMs (12 h, 36 h and 72 h ACM) compared with N-ACM, and the difference between 36 h (39.78 ± 3.0) or 72 h ACM (37.86 ± 3.1)

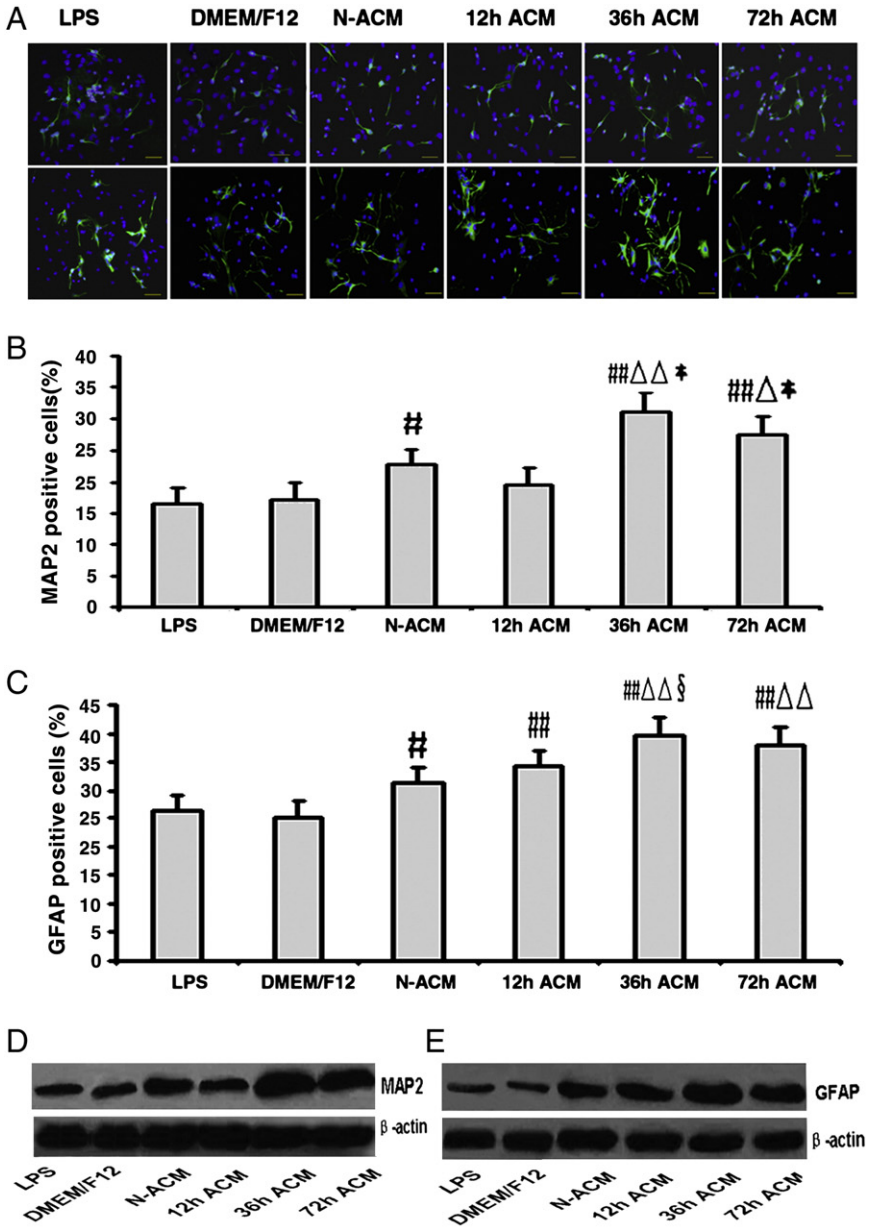

Fig. 2 ACM promotes neuronal and astrocytic differentiation of NSCs. Cells were cultured in LPS-containing DMEM/F12 (LPS), DMEM/F12, N-ACM, 12 h, 36 h, and 72 h ACM, respectively, for 7 days followed by immunostaining with anti-MAP2 and GFAP antibody (green) and counterstained with DAPI (blue). A) Showing the representative immunofluorescence images of MAP2 and GFAP-positive cells in different medium groups, respectively. Scale bar: 100 μm. B–C) Quantitative analysis of MAP2 (B) and GFAP (C)-positive cells over the total DAPI cells. Data are presented as mean ± SD ($n = 5$). D–E) Western blotting results show the expression level of MAP2 (D) and GFAP (E) protein in NSCs induced by different ACMs. β-actin serves as an internal control. #$P<0.05$, ##$P<0.01$ vs DMEM/F12; Δ $P<0.05$, Δ Δ $p<0.01$ vs N-ACM; *$P<0.01$ vs 12 h ACM; §$P<0.05$ vs 12 h ACM.

and N-ACM (31.26 ± 2.6) was statistically significant ($p<0.01$). The frequency of GFAP-positive NSCs in 36 h ACM group was significantly higher than that of 12 h ACM (34.17 ± 2.9, $p<0.05$). Western blot results (Fig. 2E) showed that GFAP protein expression in 12 h, 36 h and 72 h ACM groups was stronger than N-ACM, respectively, and GFAP expression in 36 h ACM group was the highest among different inflammatory ACM groups. Besides, there was also no significant difference on NSC's astrocytic differentiation between LPS- and DMEM/F12-treated NSCs (26.45 ± 2.7 vs 25.37 ± 2.8)(Cacci et al., 2008; Monje et al., 2003).

Bioactive molecules secreted from the astrocyte cultures

The foregoing data implicated that molecules produced by activated astrocytes can modulate the fate specification of

neural stem cells. To determine the possible factors in ACM that contribute to NSC proliferation and differentiation, proinflammatory cytokines interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) and trophic factor brain-derived neurotrophic factor (BDNF) were quantified in ACMs by ELISA. As shown in Fig. 3, the levels of IL-6, IL-1β, TNF-α and BDNF expression were all strongly elevated in inflammatory ACMs collected from LPS-stimulated astrocytes compared with N-ACM ($p<0.01$). Moreover, the amount of IL-6 in 36 h ACM (1745±213) was significantly greater than that in 12 h ACM (1256±187, $p<0.05$, Fig. 3A). In addition, the amount of TNF-α in 36 h ACM (1254±221) was significantly greater than that in 72 h ACM (736±186, $p<0.05$, Fig. 3B). Besides, the expression of IL-1β in both 36 h ACM (1752±221) and 72 h ACM (1822±213) was significantly greater than that in 12 h ACM (390±45, $p<0.01$, Fig. 3C). However, there was no significant difference in the production of BDNF in different inflammatory ACMs (12 h, 36 h, and 72 h ACMs) (Fig. 3D).

IL-6 participates in ACM-induced proliferation of NSCs

It has been reported that IL-6 expression is significantly increased in various CNS disorders and plays pleiotropic functions during the processes (Acalovschi et al., 2003; Nakamura et al., 2005). Remarkably, the pattern changes of IL-6 expression in different ACMs as described earlier appear to coincide with the effects of different ACMs on the NSCs, suggesting that the cytokine may contribute to the proliferation and differentiation of NSCs exposed to different ACMs. Furthermore, some previous results have shown the contradictory effects of IL-6 on the behaviors of NSCs (Monje et al., 2003; Barkho et al., 2006). To elucidate the roles of IL-6 on NSCs, we examined the effects of IL-6 in different ACMs on NSCs. Exogenous anti-IL-6 antibody and recombinant IL-6 cytokine were added into different ACMs 1 h prior to their use for NSC treatment. The dose of IL-6 and IL-6 antibody was first determined by the concentration gradients which showed that recombinant IL-6 cytokine at 50 ng/ml and IL-6 antibody at 30 ng/ml was optimal (data not shown). The effects of astrocyte-derived IL-6 on the proliferation of NSCs were then investigated. As shown in Fig. 4, following neutralization of IL-6 with anti-IL-6 antibody, the percentage of BrdU-positive cells was reduced compared with the corresponding control (except for 12 h ACM). The reduction was statistically significant in 36 h (24.83±2.5 vs 20.77±2.3) and 72 h (22.67±2.6 vs 18.56±2.3) ACM group ($p<0.05$). In contrast, addition of recombinant IL-6 cytokine significantly increased the proportion of BrdU-positive cells in NSCs compared with DMEM/F12 medium alone (12.58±2.4 vs 16.41±2.3, $p<0.05$).

IL-6 inhibits the neurogenesis of NSCs induced by ACM

Several *in vitro* studies have demonstrated that IL-6 participates in modulating the differentiation of NSCs (Monje et al., 2003; Taga and Fukuda, 2005; Islam et al., 2009). To investigate whether IL-6 secreted from LPS-activated astrocytes affects the neuronal differentiation of ACMs-treated NSCs, anti-IL-6 antibody or recombinant IL-6 cytokine was added into different media. The differentiation of NSCs was examined by immunocytochemistry and Western blotting methods. Results of immunostaining analysis (Fig. 5A) showed that neutralization of IL-6 with anti-IL-6 antibody increased ACM-induced neuronal differentiation of NSCs in different ACMs. The increase both in 36 h (30.49±2.6 vs 35.41±2.5) and 72 h (27.07±2.5 vs 31.75±2.4) ACM groups was statistically significant compared with the corresponding control, respectively ($p<0.05$). In contrast, after adding recombinant IL-6 cytokine into the DMEM/F12 medium, the percentage of MAP2-positive NSCs was

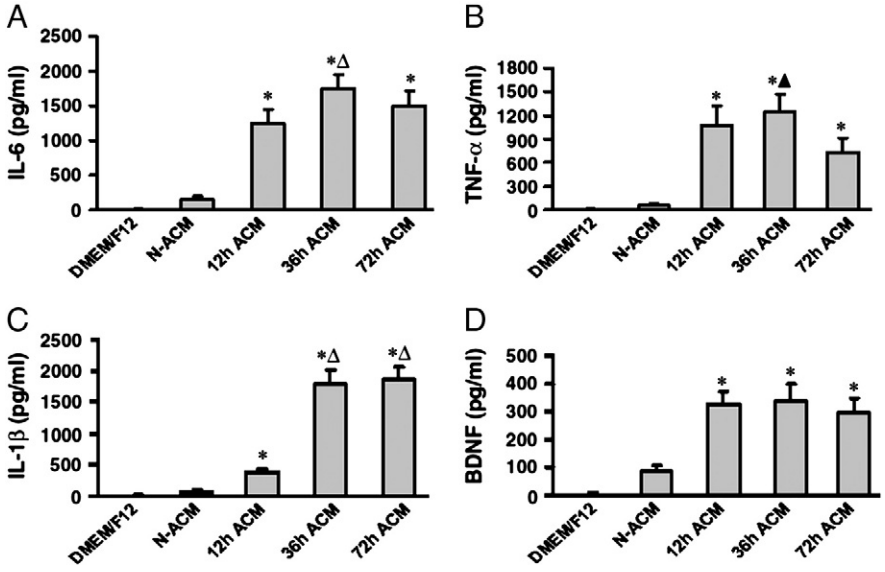

Fig. 3 Analysis of proinflammatory cytokines IL-6, TNF-α, and IL-1β, and trophic factor BDNF expression. Results of ELISA show the amounts of serial molecules such as IL-6 (A), TNF-α (B), IL-1β (C) and BDNF (D) collected from DMEM/F12 or astrocytes unstimulated or stimulated by LPS for 12 h, 36 h or 72 h, respectively. Each bar represents mean±SD ($n=3$). *$p<0.01$, vs N-ACM; Δ $p<0.01$,vs 12 h ACM; ▲$p<0.01$,vs 72 h ACM.

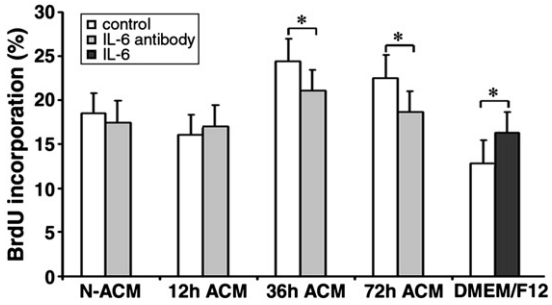

Fig. 4 IL-6 affects ACM-induced proliferation of NSCs. NSCs were cultured in N-ACM, 12 h, 36 h, and 72 h ACM, respectively, in the presence of anti-IL-6 antibody or the lack of it (control). Along with this, NSCs were cultured in DMEM/F12 medium with IL-6 cytokine. After treatment for 2 days, the NSCs were incubated with BrdU for 6 h. Quantitative analysis of BrdU-positive NSCs shows the proliferation changes of NSCs after IL-6 antibody neutralization or adding IL-6. Data are presented as mean ± SD ($n=5$). *$p<0.05$.

significantly decreased (17.14 ± 2.4 vs 13.16 ± 2.3, $p<0.05$). The results of immunocytochemistry were consistent with Western blot analysis (Fig. 5B,C) which showed that the protein expression of MAP2 in both 36 h (0.88 ± 0.07 vs 1.07 ± 0.08) and 72 h (0.74 ± 0.08 vs 0.91 ± 0.07) ACM groups was significantly stronger than the corresponding control, respectively ($p<0.05$). The MAP2 level was significantly reduced after NSCs were treated with recombinant IL-6 (0.34 ± 0.06 vs 0.21 ± 0.05, $p<0.05$). Moreover, addition of exogenous IL-6 cytokine into the 36 h ACM significantly reduced the percentage of MAP2 positive cells (29.76 ± 2.5 vs 25.16 ± 2.4, $p<0.05$).

IL-6 promotes the astrogliogenesis of NSCs induced by ACM

To further confirm whether IL-6 is involved in the astrocytic differentiation of ACM-induced NSCs, we examined the expression of GFAP in these cells. As shown in Fig. 5D, IL-6 antibody neutralization reduced the astrocytic differentiation of ACM-induced NSCs. The reduction was statistically significant between 12 h (33.95 ± 2.6 vs 28.69 ± 2.4), 36 h (39.87 ± 2.5 vs 34.64 ± 2.7) or 72 h (37.76 ± 2.6 vs 32.27 ± 2.5) ACM and the

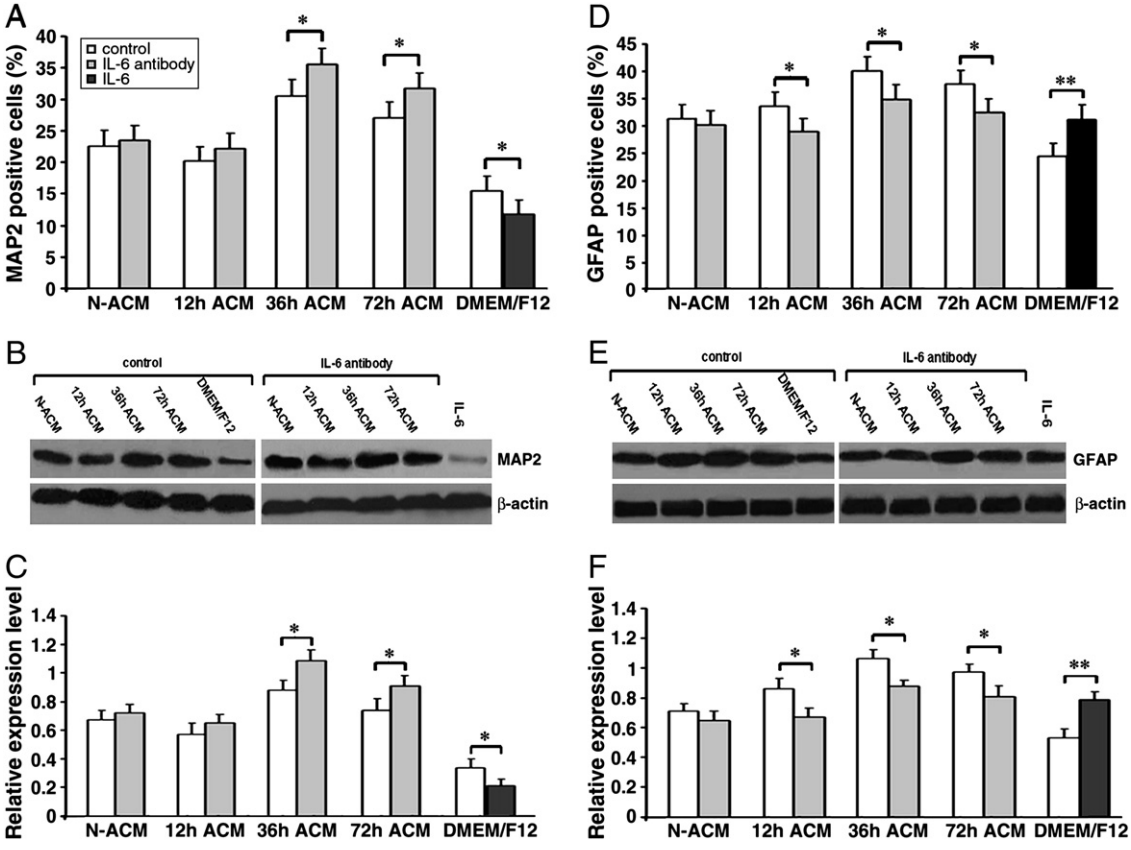

Fig. 5 IL-6 affects ACM-induced neuronal and astrocytic differentiation of NSCs. NSCs were cultured in N-ACM, 12 h, 36 h, 72 h ACM, respectively, with 1% FBS in the presence of anti-IL-6 antibody or the lack of it. In parallel to this, NSCs were cultured in DMEM/F12 medium with 1% FBS in the presence of IL-6 cytokine. After treatment for 7 days, the NSCs were processed for different experiments and analyses. A,D) Quantification of immunostaining analysis shows the changes of MAP2(A) and GFAP(D)-positive cells over the total cells in NSCs after neutralization or addition of IL-6. B,E) Western blot shows the expression level of MAP2(B) and GFAP(E) protein in different ACM-induced NSCs. β-actin serves as the internal control. C,F) Quantification of the protein expression of MAP2 (C) and GFAP (F) in NSCs shows the changes of neuronal and astrocytic differentiation of NSCs. Each bar represents mean ± SD ($n=5$). *$p<0.05$, **$p<0.01$.

corresponding control, respectively ($P<0.05$). Meanwhile, addition of recombinant IL-6 cytokine significantly increased GFAP-positive cells in NSCs (24.23±2.4 vs 31.04±2.6, $P<0.01$). Moreover, Western blot analysis (Fig. 5E,F) showed that the protein expression of GFAP was decreased after IL-6 neutralization in different ACMs [12 h (0.86±0.07 vs 0.67 ±0.08), 36 h (1.04±0.06 vs 0.87±0.04), 72 h ACM (0.97±0.05 vs 0.81±0.06, $P<0.05$]. GFAP expression was significantly increased after adding recombinant IL-6 into the DMEM/F12 medium (0.53±0.06 vs 0.79±0.05, $P<0.01$). Besides, addition of IL-6 cytokine into the 36 h ACM also significantly increased the percentage of GFAP positive cells (39.46±2.3 vs 44.57±2.4, $p<0.05$).

Stat3 signal participates in IL-6-induced proliferation of NSCs treated with ACMs

It has been reported that Janus-activated kinase/signal transducer and activation of transcription 3 (JAK/Stat3) signaling pathway plays crucial roles in the fate determination of NSCs (Gu et al., 2005; Cao et al., 2010). In light of this, we investigated whether Stat3 is involved in ACM-induced cell proliferation and differentiation of NSCs. As depicted in Fig. 6, ACMs induced the phosphorylation of an 80-kDa protein specifically recognized by anti-phospho-Stat3 (p-Stat3) antibody during NSCs proliferation. The expression of p-Stat3 (tyr705) in NSCs exposed to 12 h, 36 h, 72 h ACM was significantly enhanced compared with N-ACM, respectively. However, the total Stat3 protein expression of NSCs appeared to be unaltered when exposed to different ACMs. To further ascertain the function of Stat3 in ACM-induced NSCs proliferation, anti-IL-6 antibody or recombinant IL-6 cytokine was added into the different media, respectively. The results showed that compared with the corresponding control, the protein level of p-Stat3 (tyr705) was significantly increased after adding recombinant IL-6 cytokine (0.17±0.05 vs 0.33±0.04, $P<0.05$), while its expression was decreased after adding the IL-6 antibody, especially in 12 h (0.42±0.05 vs 0.30±0.04,), 36 h (0.43±0.06 vs 0.31±0.04,) and 72 h (0.46±0.05 vs 0.35±0.03) ACM groups, and the reduction is statistically significant, respectively ($P<0.05$). Furthermore, results about the expression change of p-Stat3 (tyr705) were also obtained during NSC differentiation (data not shown). The aforementioned data support that Stat3 is involved in NSC proliferation and differentiation induced by astrocyte-derived IL-6.

bHLH transcription factors participate in IL-6-induced differentiation of NSCs treated with ACMs

It is well documented the basic helix–loop–helix transcription factors (bHLH) are highly expressed by NSCs and positively or negatively regulate the neurogenesis and astrogliogenesis (Kageyama et al., 2005; Cao et al., 2006). To determine whether bHLH transcription factors are involved in the differentiation of ACM-induced NSCs, RT-PCR analyses were conducted to examine mRNA levels of proneuronal bHLH transcription factors (Mash1 and Neurog1) and inhibitory bHLH transcription factors (Hes5 and Hes1) in differentiated NSCs. The results (Fig. 7) showed that following IL-6 neutralization, mRNA levels of Mash1 and Neurog1 increased. The increase in Mash1 mRNA expression (Fig. 7A,B) was statistically significant in 12 h

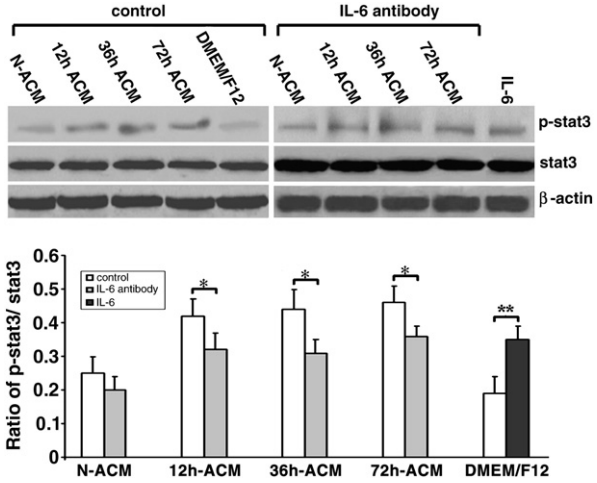

Fig. 6 Stat3 is involved in IL-6-induced proliferation of NSCs exposed to ACMs. NSCs were cultured in N-ACM, 12 h, 36 h, 72 h ACM, respectively, with 5 ng/ml bFGF in the presence or absence (control) of anti-IL-6 antibody. Meanwhile, NSCs were cultured in DMEM/F12 medium with 5 ng/ml bFGF in the presence of IL-6. A) Western blot shows the protein expression of p-stat3 (tyr705) and total stat3 in NSCs exposed to different media. β-actin serves as the internal control. B) Quantitative analysis of expression level of p-stat3 (tyr705) in NSCs shows the expression changes of p-stat3 (tyr705) in NSCs. Data are presented as mean±SD ($n=4$). *$p<0.05$, **$p<0.01$.

(0.41±0.03 vs 0.52±0.05), 36 h (0.50±0.06 vs 0.64±0.04) and 72 h (0.47±0.05 vs 0.58±0.03) ACM groups compared with the corresponding control, respectively ($P<0.05$). Similarly, Neurog1 mRNA levels (Fig. 7D,E) were significantly increased after IL-6 neutralization in 36 h (0.48±0.05 vs 0.61±0.04) and 72 h (0.45±0.03 vs 0.55±0.04) ACM groups compared with the corresponding control, respectively ($P<0.05$). However, the anti-IL-6 antiserum significantly reduced the mRNA expression of Hes5 (Fig. 7A,C) and the difference between 12 h (0.47±0.05 vs 0.37±0.04), 36 h (0.54±0.06 vs 0.43±0.04) and 72 h (0.50±0.05 vs 0.40±0.03) ACM group and the corresponding control was statistically significant, respectively ($p<0.05$). Contrary to anti-IL-6 antiserum, recombinant IL-6 cytokine significantly increased the mRNA expression level of Hes5 (0.28±0.05 vs 0.37±0.04, $p<0.05$), while reduced the mRNA expression of Mash1 (0.24±0.05 vs 0.14±0.04) and Neurog1 (0.27±0.03 vs 0.15±0.03), respectively ($p<0.05$). Interestingly, IL-6 neutralization or IL-6 cytokine did not significantly affect the expression of Hes1 mRNA (Fig. 7D,F). The data suggest that enhanced or reduced expression of these transcription factors may contribute to the increased or decreased neurogenesis and astrogliogenesis caused by astrocyte-derived IL-6.

Discussion

This study investigated the effects of inflammation-activated astrocytes at different stages on the proliferation and differentiation of NSCs as well as the possible molecular mechanisms involved in the processes. We showed that inflammatory ACMs promoted the proliferation and differentiation of NSCs, notably the 36 h ACM which exerted a greater effect on NSCs than other ACMs. Moreover, we

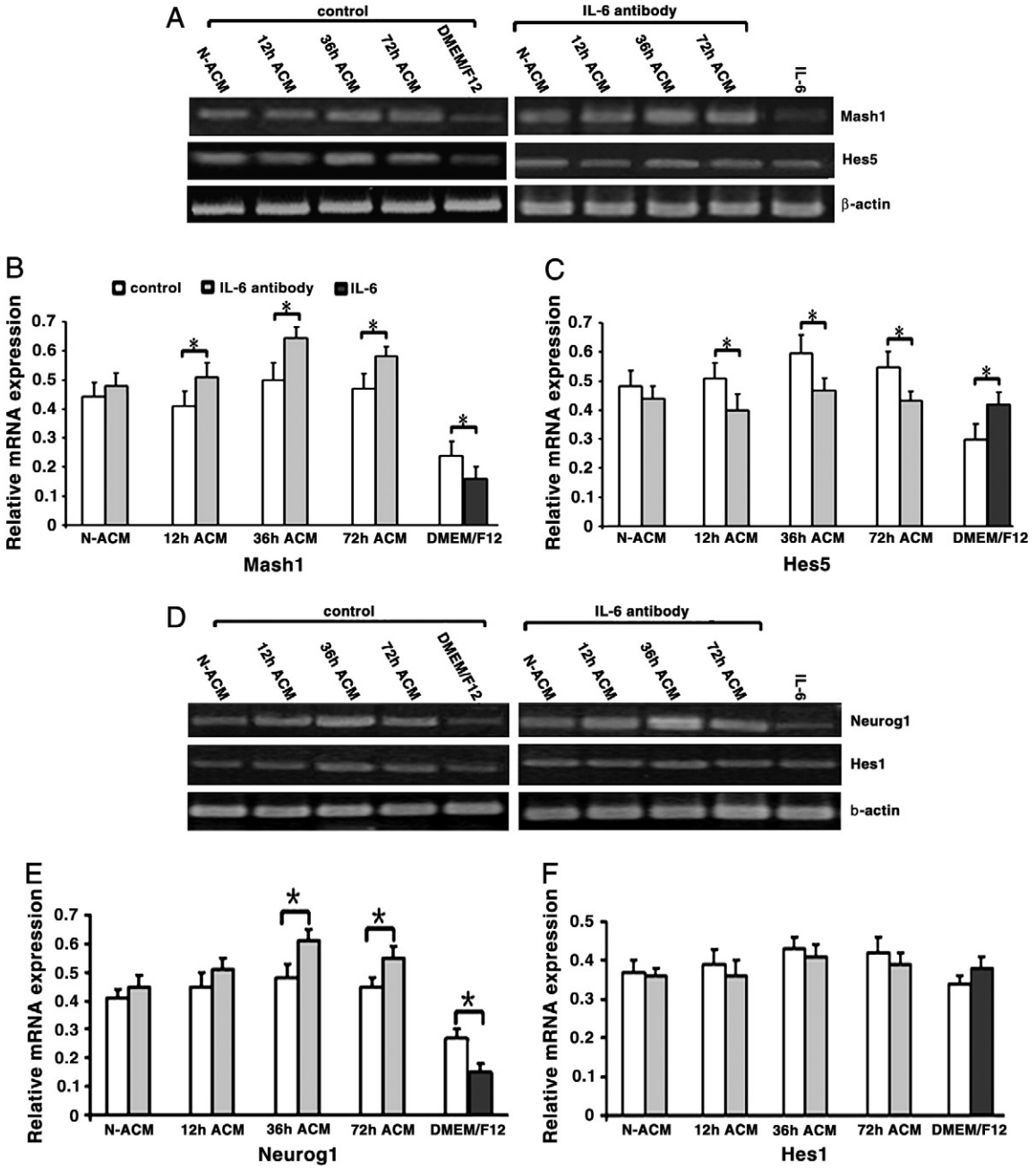

Fig. 7 bHLH transcription factors participate in IL-6-induced neural differentiation of NSCs exposed to ACMs. The NSCs were cultured in N-ACM, 12 h, 36 h, 72 h ACM, respectively, with 1% FBS in the presence or absence (control) of anti-IL-6 antibody. Meanwhile, NSCs were cultured in DMEM/F12 medium with 1% FBS in the presence of IL-6. RT-PCR analyses show the mRNA expression levels of bHLH transcription factors, Mash1 Neurog1, Hes5 and Hes1. β-actin serves as the internal control. Values are presented as mean ± SD from four independent measurements. $^*p<0.05$.

identified astrocyte-derived IL-6 as a molecule important for ACM-induced proliferation and differentiation via the phosphorylation of Stat3 signaling pathway. In addition, the bHLH transcription factors such as Mash1, Neurog1 and Hes5 also participate in the ACM-induced NSCs differentiation.

Accumulating evidence has demonstrated that astrocytes play important roles in modulating the proliferation and differentiation of NSCs (Ma et al., 2005; Song et al., 2002) However, little is known on how activated astrocytes under inflammatory CNS circumstances regulate the behavior of NSCs. The present results have shown that compared with N-ACM, 36 h and 72 h ACM significantly promoted proliferation and differentiation of NSCs (Figs. 1 and 2), suggesting that inflammation-activated astrocytes exerted greater effects in cell fate determination of NSCs than unstimulated astrocytes. It has been reported that in response to stimulation, astrocytes significantly upregulate the production of a plethora of molecules, including pro- and anti-inflammatory cytokines, growth factors and trophic factors (Ridet et al., 1997; Lieberman et al., 1989; Lafon-Cazal et al., 2003; Vesce et al.,

2007). Most of the secreted factors, such as IL-6, TNF-α, retinoic acid, nerve growth factor and ciliary neurotrophic factor (CNTF), have been demonstrated to participate in the proliferation and differentiation of NSCs (Ma et al., 2005; Cattaneo and McKay, 1990; Bonni et al., 1997; Ricci-Vitiani et al., 2006; Környei et al., 2007; Johansson et al., 2008). It would appear therefore that the increased factors from activated astrocytes in combination might form a special stem cell microenvironment that may facilitate the proliferation and differentiation of NSCs. Horner and others have reported that astrocytes activated by ischemia or mechanical-lesion significantly accelerate the proliferation and differentiation of neural stem/progenitor cells (Faijerson et al., 2006; Horner and Palmer, 2003). Taken together, it is suggested that optimally activated astrocytes under inflammatory CNS conditions may promote the proliferation and differentiation of NSCs.

The present study has extended our previous observations (Wang et al., 2009) that ACMs derived from astrocytes stimulated by LPS for different duration exerted distinct roles on NSCs. Previous studies showed that the factors produced by activated astrocytes varied considerably with the nature, intensity and persistence of stimulation (Tokumine et al., 2003). It was reported that different molecules or the same factors but of different concentrations would have distinct effects on the behavior of stem cells. For example, Chang et al. (2003) reported that the effects of bone morphogenetic protein (BMP) on NSCs are dose-dependent, namely, low doses of BMP stimulated cortical NSCs to exclusively differentiate into neurons, while astrocyte differentiation was induced when higher doses of BMPs were used. Besides, CNTF and other molecules with different concentrations were also reported to have distinct effects on the fate specification of NSCs (Qian et al., 1997; Bhattacharya et al., 2008). Considering the pivotal effects of local microenvironment on stem cells (Ma et al., 2005; Ricci-Vitiani et al., 2006; Constantinescu, 2000; Temple, 2001), it is suggested that activated astrocytes at various stages may have distinct roles in regulating the behavior of stem cells by releasing different factors. This should be taken into consideration in designing and selection of time point for an effective NSC transplantation therapy.

It has been reported that the bioactive molecules produced by astrocytes participate in regulating the behavior of NSCs (Ma et al., 2005; Song et al., 2002). Our results have shown that several proinflammatory cytokines including IL-6, TNF-α and IL-1β, and trophic factor BDNF are secreted from stimulated and unstimulated astrocytes (Fig. 3). Identification of the molecular cues that modulate NSCs fate choice is a prerequisite for their therapeutic applications. Among the various molecules, IL-6, a pleiotropic inflammatory factor, was systematically investigated in the present study. This takes into account of the vigorous activation of astrocytes in the damaged CNS and the fact that IL-6 expression is significantly increased in various CNS disorders and that it participates in the proliferation and differentiation of stem cells (Acalovschi et al., 2003; Nakamura et al., 2005; Taga and Fukuda, 2005). In the present study, ELISA results have shown that IL-6 expression was significantly up-regulated in inflammatory ACMs from activated astrocytes and the amounts of IL-6 secreted in 12 h, 36 h and 72 h ACM varied according to the duration of LPS stimulation. The varied expression changes of IL-6 suggest that the cytokine may contribute to the proliferation and differentiation of varying magnitude in NSCs exposed to different ACMs. This notion lends its support from the fact that NSCs changed their behaviors swiftly with IL-6 antibody neutralization or exogenous application of IL-6 in the medium. This is in agreement with earlier studies which reported that IL-6 participates in modulating the proliferation and differentiation of NSCs (Taga and Fukuda, 2005; Islam et al., 2009). Vallières et al. reported that hippocampal neurogenesis was significantly reduced in adult transgenic mice with chronic astrocytic production of IL-6 (Vallières et al., 2002). Additionally, it has been reported that astrocytic differentiation of NSCs was apparently decreased with the blockade of the action of IL-6 receptor (Okada et al., 2004). In addition to modulating the differentiation of NSCs, IL-6 is also reported to be involved in NSC proliferation (Kang and Kang, 2007). In consideration of our present results along with others (Wang et al., 2009; Barkho et al., 2006), it is suggested that IL-6 serves as an important molecule in regulating the ACM-induced proliferation and differentiation of NSCs. Further studies using IL-6 gene knockout mice or injecting IL-6 cytokine or IL-6 antibody *in vivo* may allow us to decipher the role of IL-6 in response to various CNS diseases.

Previous studies have reported that IL-6 binds to the heterodimeric complex gp130/IL-6 receptors, followed by the activation of JAK/STAT pathway (Gu et al., 2005; Heinrich et al., 1998; Bauer, 2009). The activated Stat3 then translocates into the nucleus, regulates the expression of different transcription factors and plays various biological effects, such as cell survival, apoptosis, proliferation, migration and differentiation (Gu et al., 2005; Kang and Kang, 2007; Schindler and Darnell, 1995; Ihle, 2001; Iwamaru et al., 2007). Recently, there is increasing evidence that JAK/STAT signaling pathway plays crucial roles in the determination of the fate of NSCs. For instance, some studies have reported that inhibition of Stat3 activity suppresses astrogliogenesis and promotes neurogenesis in cultured NSCs (Gu et al., 2005; Cao et al., 2006). The present results have shown that the expression of p-stat3 was apparently increased in NSCs induced by inflammatory ACMs during NSC proliferation. More importantly, the expression was significantly changed with the neutralization of IL-6 or with the addition of exogenous IL-6 cytokine, respectively. Besides, the expression of p-Stat3 during NSC differentiation is similar to that during NSC proliferation in our culture conditons (data not shown), which is consistent with previous reports that JAK/STAT3 participated in the differentiation of NSCs (Gu et al., 2005; Cao et al., 2010; Bonni et al., 1997). Arising from this, it is suggested that IL-6 modulates the ACM-induced proliferation and differentiation of NSCs via the phosphorylation of Stat3 signaling at least.

Despite the many studies in recent years on neurogenesis and gliogenesis, the precise mechanisms by which they are regulated in the NSCs remain to be elucidated. In addition to the JAK/STAT3 signaling pathway we discussed earlier, accumulating data have shown that the bHLH transcription factors, play important roles in the determination of neuron and glia cell fates from NSCs during fetal and adult brain development (Kageyama et al., 2005; Ross et al., 2003; Havrda et al., 2008). Several studies have shown that the proneural bHLH transcription factors such as Mash1, Neurog1, and NeuroD2 participate in the vertebrate neurogenesis and increase in Mash1 and Neurog1 expression leads to the initiation of neurogenesis. On the other hand, upregulated

expression of inhibitory transcription factors Hes and Id increased astrogliogenesis (Nakamura et al., 2001; Ito et al., 2003; Sugimori et al., 2007). We show here the involvement of Mash1, Neurog1 and Hes5 in the ACM-induced neuronal and astrocytic differentiation of NSCs. In this connection, ACMs markedly induced upregulation of Mash1, Neurog1 and Hes5 mRNA in differentiated NSCs. In addition, exogeneous application of IL-6 antibody or recombinant IL-6 cytokine significantly affected the mRNA expression levels of the transcription factors, suggesting that the neurogenesis or astroglionenesis of NSCs caused by ACMs is partially attributed to the expression of these transcription factors. However, in the present study, there was no significant expression change in Hes1 mRNA when the NSCs were exposed to different ACMs, and IL-6 neutralization or addition of IL-6 cytokine did not also significantly affect the expression of Hes1 mRNA, suggesting that Hes1 may not participate in IL-6-induced differentiation of NSCs treated with ACMs.

Although IL-6 in the ACMs participates in the proliferation and differention of NSCs as shown in the present study, it needs to be emphasized that IL-6 neutralization did not completely block ACM-induced NSC proliferation and differentiation compared with DMEM/F12 medium alone. This suggests that apart from IL-6, other molecules secreted by activated astrocytes may be involved in the process. As shown in Fig. 3, the production of other proinflammatory cytokines such as TNF-α and IL-1β, and trophic factor BDNF from astrocytes has also increased significantly. Furthermore, consistent with the findings by others (Liu et al., 2005; Peng et al., 2008), TNF-α participates in the proliferation and differentiation of NSCs (data not shown). In addition, BDNF, IL-1β, BMP, CNTF, nerve growth factor; and retinoic acid derived from astrocytes were also confirmed to participate in modulating the fate determination of NSCs (Cattaneo and McKay, 1990; Környei et al., 2007; Chang et al., 2003; Nakanishi et al., 2007). All these indicate that IL-6 along with other molecules derived from astrocytes may act in concert either to facilitate or inhibit the behaviors of NSCs. The combined actions of these bioactive molecules on NSCs are very complex and await further exploration both *in vitro* and *in vivo*.

Materials and methods

The International Guiding Principles for Animal Research, as stipulated by the World Health Organization (1985) (Howard-Jones, 1985) and as adopted by the Laboratory Animal Center, Shandong University were followed. All efforts were made to reduce the number of mice and their suffering.

Astrocyte culture and preparation of conditioned medium

Primary astrocytes were prepared from 1-day-old KunMing mice as described before (McCarthy and de Vellis, 1980) with modifications. Briefly, the cell suspension of cortical tissues freed of meninges and blood vessels was seeded in DMEM/F12 medium (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA). After incubating for 10 days, to obtain pure astrocytes, the primary mixed cells were orbitally shaken at 200 rpm for 6 h and the supernatant was removed. The attached cells in the plate were washed with phosphate-buffered saline, digested by trypsin and then replated. The purity of the cultures was assessed by indirect immunofluorescence for the astrocytic specific marker (glial fibrillary acidic protein, GFAP, Chemicon). On average, 96.2 ± 0.6% of the cells were positive for the expression of GFAP. When the astrocytes have reached about 80% of confluence at passage 3, the cells were treated with fresh DMEM/F12 plus 1 μg/ml LPS (from Escherichia Coli, serotype 026:B6, Sigma-Aldrich, USA) for 12, 36 and 72 h, respectively. After stimulation, the supernatant was collected, respectively, to obtain the inflammatory astrocyte-conditioned medium (ACM) at the foregoing time points designated as 12 h ACM, 36 h ACM and 72 h ACM. Meanwhile, the supernatant from astrocytes treated with fresh DMEM/F12 alone for 36 h was collected to be used as normal ACM (N-ACM). The ACMs were centrifuged, filtered and stored at −80 °C until tested or later use.

Neural stem cell cultures

The primary culture of NSCs was prepared from embryos (E13.5) of KunMing mice as described previously (Fu et al., 2006). Briefly, the telencephalon was dissected out from the embryo and the cultures were incubated in the growth medium consisting of DMEM/F12 supplemented with basic fibroblast growth factor (bFGF) (20 ng/ml, Gibco Invitrogen Corporation), epidermal growth factor (20 ng/ml, Gibco Invitrogen Corporation) and 2% B27 (Gibco Invitrogen Corporation) (growth medium). The culture medium was changed every 2 days and the cells grew into floating neurospheres. After 7 days *in vitro*, primary neurospheres were dissociated into single cells and the cells grew into neurospheres again. Secondary or tertiary neurospheres were used for subsequent experiments and all experimental procedures were carried out using monolayers of NSCs.

Neural stem cells viability analysis

Cell viability of NSCs was determined by the tetrazolium salt MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay. Dissociated NSCs were seeded at a density of 5×10^3 cells/ml in poly-L-lysine (Sigma-Aldrich, USA)-coated 96-well culture plate and cultured in NSC growth medium for 24 h. NSCs were then exposed to N-ACM, 12 h ACM, 36 h ACM and 72 h ACM (without dilution), respectively, with 5 ng/ml of bFGF. After 48 h of incubation, MTT solution (5 mg/ml) was added to each well and incubated at 37 °C for 4 h. The medium was gently aspirated and 200 μl dimethylsulfoxide was added to each well. The optical density of each sample was immediately measured in a multiwell spectrophotometer (BioRad, USA) at 490 nm. Separate experiments were conducted four times.

Proliferation index by bromodeoxyuridine (BrdU) labeling and immunostaining

To examine the proliferation, dissociated NSCs were plated on coverslips pre-coated with poly-L-lysine in six-well plates and cultured in NSC growth medium for 24 h. Then, the NSCs were treated with N-ACM, 12 h ACM, 36 h ACM and 72 h ACM (without dilution), respectively, with 5 ng/ml of bFGF for 2 days in the absence or presence of 30 ng/ml anti-IL-6 antibody (Chemicon, USA). In addition, NSCs were exposed to

DMEM/F12 midium with 5 ng/ml of bFGF for 2 days with or without recombinant 50 ng/ml IL-6 cytokine (RD, USA). BrdU (10 μg/ml, Sigma-Aldrich) was then added to each medium for 6 h and the cell proliferation activity was estimated by BrdU incorporation analysis through immunocytochemistry. Cells were fixed in 4% paraformaldehyde for 20 min, treated with 2 mol/L HCl at 37 °C for 30 min, blocked with 5% normal goat serum for 30 min, and incubated with anti-BrdU monoclonal antibody (1:1000, Sigma-Aldrich) overnight at 4 °C. Anti-mouse IgG coupled to TRITC (1:200, Chemicon, USA) was used as the secondary antibody. Then the cells were counterstained by 4, 6-diamino-2-phenylindole (DAPI, Sigma-Aldrich, USA). The percentage of BrdU-positive cells over total DAPI cells was determined by randomly counting 10 nonoverlapping microscopy fields of three coverslips for each condition, in at least four independent experiments. An average of 100 cells per field was counted.

Neural differentiation of NSCs

Dissociated NSCs were plated on coverslips pre-coated with poly-L-lysine in six-well plates and cultured in NSC growth medium for 24 h. Following this, the NSCs were treated with N-ACM, 12 h ACM, 36 h ACM and 72 h ACM (without dilution), respectively, with 1% FBS in the absence or presence of 30 ng/ml anti-IL-6 antibody. Along with this, NSCs were exposed to DMEM/F12 medium with 1% FBS with or without recombinant 50 ng/ml IL-6 cytokine. The media were changed every 2 days. After 7 days of induction, the cells were collected and processed for immunostaining and Western blotting analysis.

Immunofluorescence assay

The induced cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.3% Triton X-100, and blocked with 5% normal goat serum. Then the cells were incubated with primary antibodies that mark microtubule-associated protein 2 (MAP2) (1:200, monoclonal, Chemicon), and GFAP (1:500, monoclonal, Chemicon) overnight at 4 °C. Subsequently, cells were incubated with FITC-conjugated secondary antibodies and counterstained with DAPI. Labelled cells were visualized and photographed with a fluorescence microscope (IX71, Olympus, Japan). Immunoreactivity was abrogated when the primary antibodies were omitted from the incubation medium. The percentage of neuron- and astrocyte-positive cells in relation to the total cells was determined in 10 independent fields for each condition in five separate experiments. An average of 100 cells per field was counted.

Western blots analysis

The induced cells were lysed and centrifuged at 14,000×*g* for 20 min. Protein concentration of the supernatants of cell extract was determined using a BCA protein assay kit (Pierce Biotechnology Inc, USA). Equivalent amounts of protein samples were loaded on SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to PVDF membranes, and the blots were subsequently probed with the following antibodies: MAP2 (1:1000), GFAP (1:1000), stat3, p-stat3) (1:1000, CST), and β-actin (1:1000, Sigma-Aldrich). For detection, horseradish peroxidase-conjugated secondary antibodies were used (1:5000) followed by enhanced chemiluminescence development (Millipore). Normalization of the results was done by running parallel Western blots using β-actin as control. The optical density was quantified using the Image-Pro Plus 6.0 software. Separate experiments were conducted four times.

Enzyme linked immuno sorbent assay (ELISA)

To identify the activation of astrocytes and explore the amounts of cytokines in different ACMs, enzyme linked immuno sorbent assay (ELISA) was adopted. The amount of proinflammatory cytokines IL-6, TNF-α, IL-1β and trophic factor BDNF in different ACMs was measured using ELISA assay, respectively (IL-6 and IL-1β kits were purchased from Genetimes, China; TNF-α and BDNF kits were purchased from R&D, USA). The procedures were strictly carried out according to the description of the ELISA kits and the optical density (OD) was measured at 450 nm in a standard microplate reader (BioRad, USA). Three independent experiments were conducted.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from NSC cultures using the Trizol (Invitrogen) method. RNA concentration was determined by a spectrophotometer at 260 nm. Identical amounts of RNA were reverse transcribed into cDNA, which was subsequently amplified by PCR with specific primers. Primers for detecting genes are listed in Table 1. The PCR products separated on 1.5% agarose/TAE gels were visualized by staining with ethidium bromide and semiquantified using AlphaEase FC Version 4 analysis software (Alphalmager HP, Alpha Innotech, USA). The densitometric analysis of the data was normalized to the β-actin. Results were mean ± SD from four separate experiments for each group.

Statistical analysis

All data were presented as mean ± SD of at least three independent experiments. Statistical analysis of data was

Table 1 Sequences and PCR product sizes for each pair of probes.

Primer	Sequence (5′→3′)	Product size (bp)
Mash1	F:AAG TCA GCG GCC AAG CAG GTC AAG R:CGC AGC GTC TCC ACC TTG CTC ATC T	245
Neurog1	F:CGA TCC CCT TTT CTC CTT TC R:TGC AGC AAC CTA ACA AGT GG	239
Hes5	F:GAT GCT CAG TCC CAA GGA GA R:CGC TGG AAG TGG TAA AGC AG	236
Hes1	F:CGA GCG TGT TGG GGA AGT A R:AGT GCG CAC CTC GGT GTT A	101
β-actin	F:AGA TGT GGA TCA GCA AGC AG R:GCG CAA GTT AGG TTT TGT CA	104

F, forward; R, reverse.

made by Student's *t*-test or one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. $P<0.05$ was considered to be statistically significant.

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